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The WH1 and EVH1 Domains of WASP and Ena/VASP Family Members Bind Distinct Sequence Motifs

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Summary

A complex of N-WASP and WASP-interacting protein (WIP) plays an important role in actin-based motility of vaccinia virus and the formation of filopodia [1, 2]. WIP is also required to maintain the integrity of the actin cytoskeleton in T and B lymphocytes and is essential for T cell activation [3, 4]. However, in contrast to many other N-WASP binding proteins, WIP does not stimulate the ability of N-WASP to activate the Arp2/3 complex [2]. Although the WASP homology 1 (WH1) domain of N-WASP interacts directly with WIP [2, 5, 6], we still lack the exact nature of its binding site. We have now identified and characterized the N-WASP WH1 binding motif in WIP in vitro and in vivo using *Shigella* and vaccinia systems. The WH1 domain, which is predicted to have a similar structural fold to the Ena/VASP homology 1 (EVH1) domain [7, 8], binds to a sequence motif in WIP (ESRFYFHPISD) that is very different from the EVH1 proline-rich DL/FPPPP ligand [9–11]. Interaction of the WH1 domain of N-WASP with WIP is dependent on the two highly conserved phenylalanine residues in the motif. The WH1 binding motif we have identified is conserved in WIP, CR16, WICH, and yeast verprolin.

Results and Discussion

Biochemical studies and extensive analysis with the yeast two hybrid system have demonstrated that WIP binds to the WH1 domain of N-WASP and WASP [1, 2, 5, 6]. The residues in WIP with which the WH1 domain interacts are, however, unknown. The strong sequence conservation between the WH1 and the EVH1 domains, including the residues involved in coordinating EVH1 binding to FPPPP ligands, suggests that the WH1 domain may also bind a proline-rich ligand [7, 8, 11, 12]. Consistent with this notion, the WASP binding domain (WBD) in WIP consists of 26% proline residues and contains four proline sequence motifs, one of which (DLPPPEP) is closely related to the VASP EVH1 binding motif [5] (Figure 1A). Furthermore, mutation of tryptophan 54 in the N-WASP WH1 domain to alanine abolishes its ability to interact with WIP [1]; an equivalent residue to that found in this domain is essential in coordinating the interaction with proline-rich sequences in the EVH1 domains of Evl and Mena [7, 8].

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To examine if the WH1 domain does indeed interact with proline-rich ligands, we sought to characterize the N-WASP binding motif in the WASP binding domain of WIP (residues 413–503). Peptides corresponding to the four proline-rich sequences in the WIP-WBD were tested for their capacity to retain the N-WASP WH1 domain from an *Escherichia coli* soluble fraction (Figures 1A and 1B). Surprisingly, all four peptides failed to show significant binding, suggesting either that the interaction site is elsewhere or that efficient WH1 binding requires a larger sequence motif than is required for the EVH1 domain. Consistent with these suggestions, recent studies with the mammalian WIP homologs CR16 and WICH have demonstrated that exon 7 of CR16 and the equivalent region in WICH, which are 50% and 47% identical to residues 448–481 of human WIP, respectively, interact with N-WASP [13, 14]. We therefore performed identical pull-down assays with a larger peptide corresponding to residues 451–473 of WIP. This larger peptide was found to be extremely efficient at binding the N-WASP WH1 domain (Figure 1B). As expected from our previous observations [1], the WH1-W54A mutant protein failed to bind residues 451–473 of WIP (data not shown). The strong binding of WH1 to residues 451–473 of WIP and the lack of significant binding to the shorter peptide (ISDLPPPEPYVQ) prompted us to wonder whether the WH1 binding site was in fact in the N-terminal half rather than the proline-rich C-terminal half of peptide E (Figure 1A). We therefore performed pull-down assays with a peptide corresponding to residues 451–461 of WIP (ES RFYFHPISD). We found that residues 451–461 of WIP (peptide F) are sufficient to efficiently bind the WH1 domain of N-WASP (Figure 1C).

Identification of the EVH1 binding motif (DL/FPPPP) was facilitated by characterization of the interaction between VASP and the proline-rich repeats of ActA, the bacterial surface protein responsible for initiating the actin-based motility of *Listeria* [9–11]. VASP binding studies have also been performed on known SH3 and WW/WP proline-rich ligands [9]. These data, however, do not rule out the possibility that the EVH1 domain of Ena/VASP family members might also bind residues 451–461 of WIP, albeit with reduced affinity. To test whether residues 451–461 of WIP are specific for the WH1 domain, we examined the binding properties of the EVH1 domain from VASP. We found that, although the VASP EVH1 domain was able to bind a control FPPPP peptide found in ActA, it was unable to bind the peptide corresponding to residues 451–461 of WIP (Figure 1D). Our data show that, although the WH1 and EVH1 domains are predicted to have a similar structural fold and are thus often grouped together, for example [9–11], they do in fact have distinct binding motifs.

The alignment of the N-WASP WH1 binding motif, which we have identified in WIP, with the corresponding regions of CR16 and WICH reveals that it is a highly conserved amino acid sequence (Figure 2A). Pull-down assays with the equivalent sequence motifs of CR16 and WICH confirmed that they are also capable of bind-

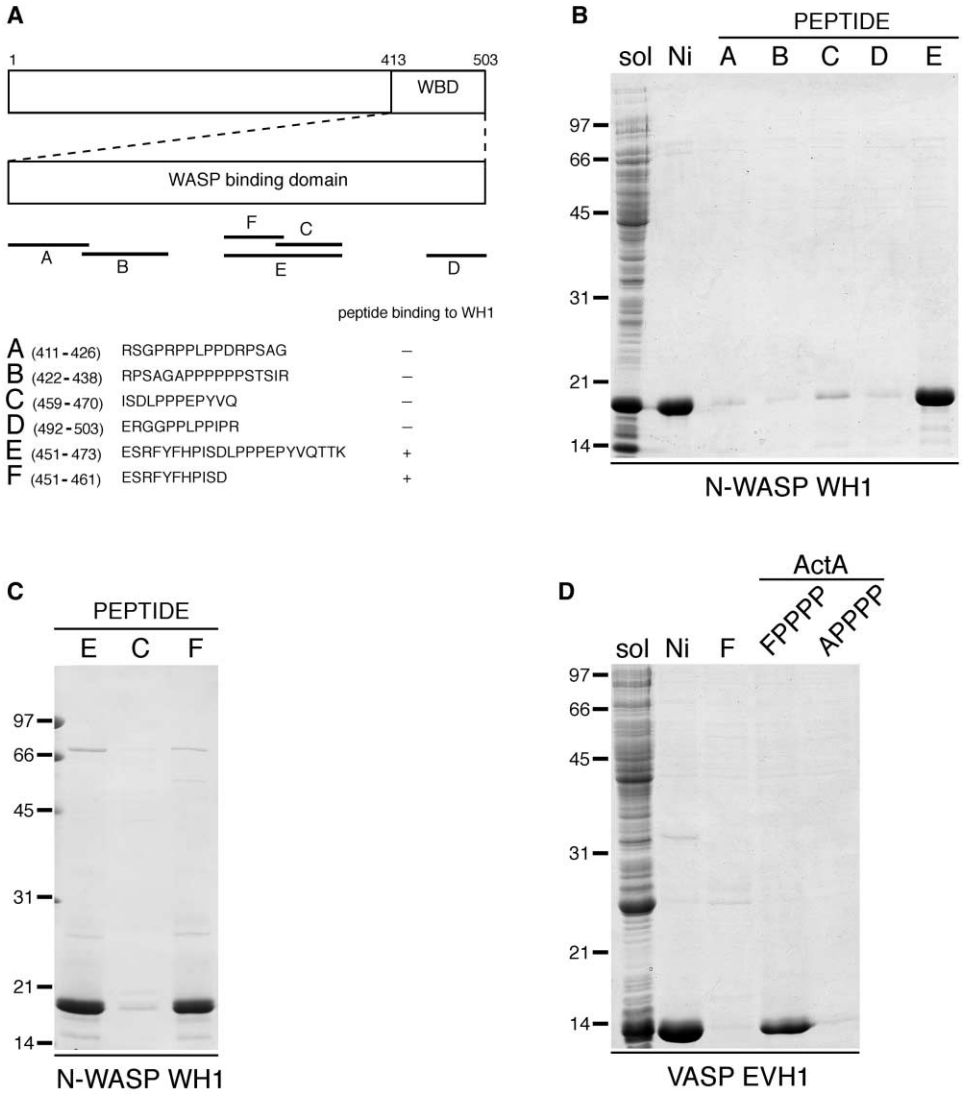

Figure 1. Identification of the WH1 Binding Site in WIP In Vitro

(A) A schematic representation of WIP showing the relative position of the WASP binding domain (WBD) and the peptides (A–F) used for in vitro binding studies. The amino acid sequence of these peptides and their N-WASP WH1 binding activity are indicated.

(B) A Coomassie-stained gel showing that the His-tagged WH1 domain of N-WASP is retained from a soluble *E. coli* extract (sol) by nickel resin (Ni) and peptide E, but not by peptides A–D.

(C) A Coomassie-stained gel showing that residues 451–461 of WIP (peptide F) are sufficient to interact with the WH1 domain of N-WASP.

(D) A Coomassie-stained gel showing that the His-tagged EVH1 domain of VASP, from a soluble *E. coli* extract (sol), binds to the nickel resin (Ni) and the positive control ActA peptide FPPPP, but not to the negative control ActA peptide APPPP or residues 451–461 of WIP (peptide F).

ing the WH1 domain of N-WASP (Figure 2B). It was noticeable, however, that the CR16 peptide consistently retained less WH1 than the other two peptides, suggesting that it has a lower affinity for N-WASP than either WIP or WICH. The WH1 binding motif is also conserved in the more divergent verprolin (End5), the WIP homolog in yeast that interacts with Las17 (Bee1), the yeast homolog of WASP [15–17]. Interestingly, extensive homology searches using the conserved WIP motif failed to identify any potential novel WH1 binding proteins, although they readily identify homologs of WIP, CR16, and WICH in other species. This suggests that the WH1 domain of N-WASP and WASP interacts with relatively few proteins, except for WIP family members, via this binding site.

To identify which residues are required for the interaction of WIP with the WH1 domain of N-WASP, we analyzed the effects of alanine substitution on the most conserved residues in the motif. We found that only changes of the two central phenylalanine residues to alanine had any noticeable affect on WH1 binding (Figure 2C). Substitution of phenylalanine 454 resulted in reduced binding, while changing phenylalanine 456 to alanine almost completely abolished the interaction with the WH1 domain. When both phenylalanine 454 and 456 were substituted for alanine, all binding was eliminated

A

human	WIP (451–461)	E	S	R	F	Y	F	H	P	I	S	D
human	CR16 (390–400)	E	S	K	F	T	F	H	S	V	E	D
human	WICH (397–407)	E	S	K	Y	S	F	H	P	V	E	D
S. cerivisiae	VRP (777–787)	D	S	R	F	K	W	T	N	V	S	Q
S. pombe	VRP (270–280)	H	G	R	F	H	F	K	D	D	S	Y
consensus		–	S	+	F	/	F	/	/	/	/	/

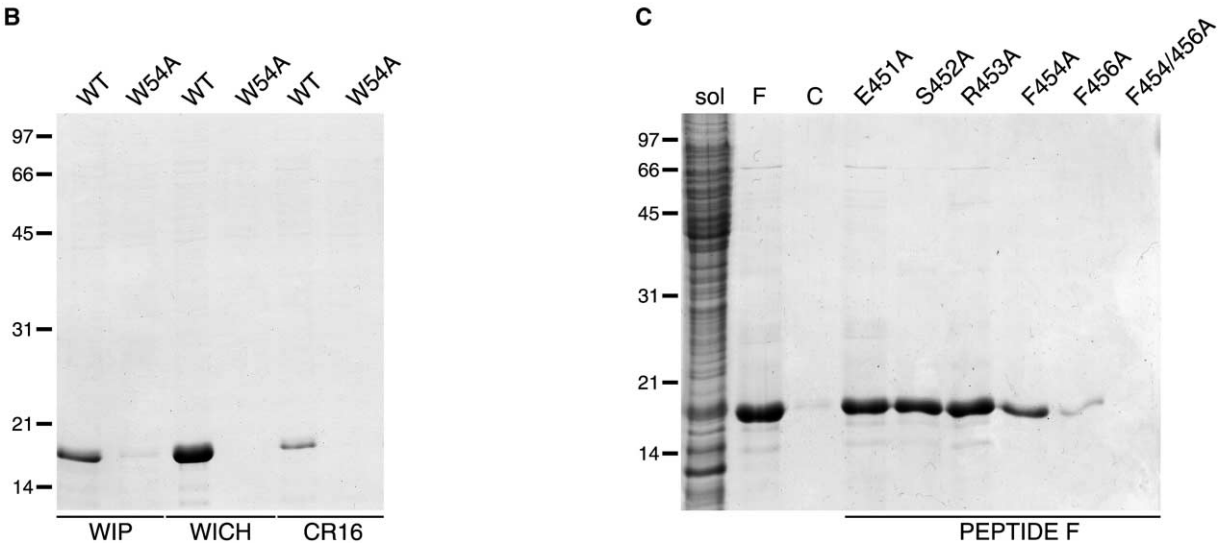

Figure 2. Phenylalanine 454 and 456 of WIP Are Involved in WH1 Binding In Vitro

(A) The alignment of the WIP homologs CR16 and WICH, which bind N-WASP, reveals that the WH1 binding motif in WIP is highly conserved. The motif is also readily observed in verprolin, the yeast homolog of WIP. The consensus shows positions at which four out of the five aligned proteins have identical residues or a conserved charge.

(B) A Coomassie-stained gel showing that the motif found in WIP, WICH, and CR16 (indicated in [A]) binds the N-WASP WH1 domain, but not the mutant WH1-W54A.

(C) A Coomassie-stained gel showing the effects of alanine substitutions on the ability of residues 451–461 of WIP (peptide F) to retain the WH1 domain from identical amounts of soluble *E. coli* extract (sol). Substitution of phenylalanine 454 and 456 for alanine leads to a reduced binding, while mutation of both residues completely abolishes the interaction.

(Figure 2C). Our in vitro binding assays demonstrate that, although the N-WASP WH1 binding motif contains a number of conserved residues, the principal residues involved in binding are the central phenylalanines.

To confirm whether our in vitro biochemical observations reflect the in vivo requirements for WIP binding to N-WASP, we took advantage of the *Shigella* and vaccinia pathogen systems. Our previous observations have shown that, when expressed in infected cells, the WIP-WBD is recruited to *Shigella* nucleating actin tails [1]. We therefore examined the ability of GFP-tagged WIP-WBD, which contains the phenylalanine 454 and 456 to alanine substitutions, to be recruited to *Shigella*. We found that GFP-WBDF454A was largely cytoplasmic but was capable of being recruited to *Shigella* nucleating actin tails on rare occasions (Figure 3A). In contrast, mutation of phenylalanine 456 to alanine resulted in a complete lack of recruitment, as did subsitution of both phenylalanine residues (Figure 3A). While recruitment provides a qualitative assay, it gives no quantitative measure of the effect of our mutations on their affinity for N-WASP in vivo. Our previous studies have, however, shown that, in contrast to the situation with *Shigella,* the expression of WIP-WBD inhibits N-WASP recruitment and subsequent actin-based motility of vaccinia [1]. Therefore, by examining the ability of the different phenylalanine-to-alanine mutations in WIP-WBD to inhibit vaccinia-induced actin tail formation, it is possible to obtain quantitative data that can be compared with our in vitro studies. Expression of wild-type GFP-WIP-WBD blocked viral recruitment of N-WASP and inhibited vaccinia-induced actin tail formation by 71.8% $\pm$ 4.8% (Figures 3B and 3C). In contrast, GFP alone did not block N-WASP recruitment and gave a 8.5% $\pm$ 1.7% reduction in actin tail formation when compared to untransfected controls on the same coverslip (data not shown and Figure 3C). Expression of GFP-WIP-WBDF454A resulted in a significant reduction in the level of inhibition to 33.6% $\pm$ 16.2%. This was reduced still further to 17.8% $\pm$ 5.2% in the case of GFP-WIP-WBDF456A and

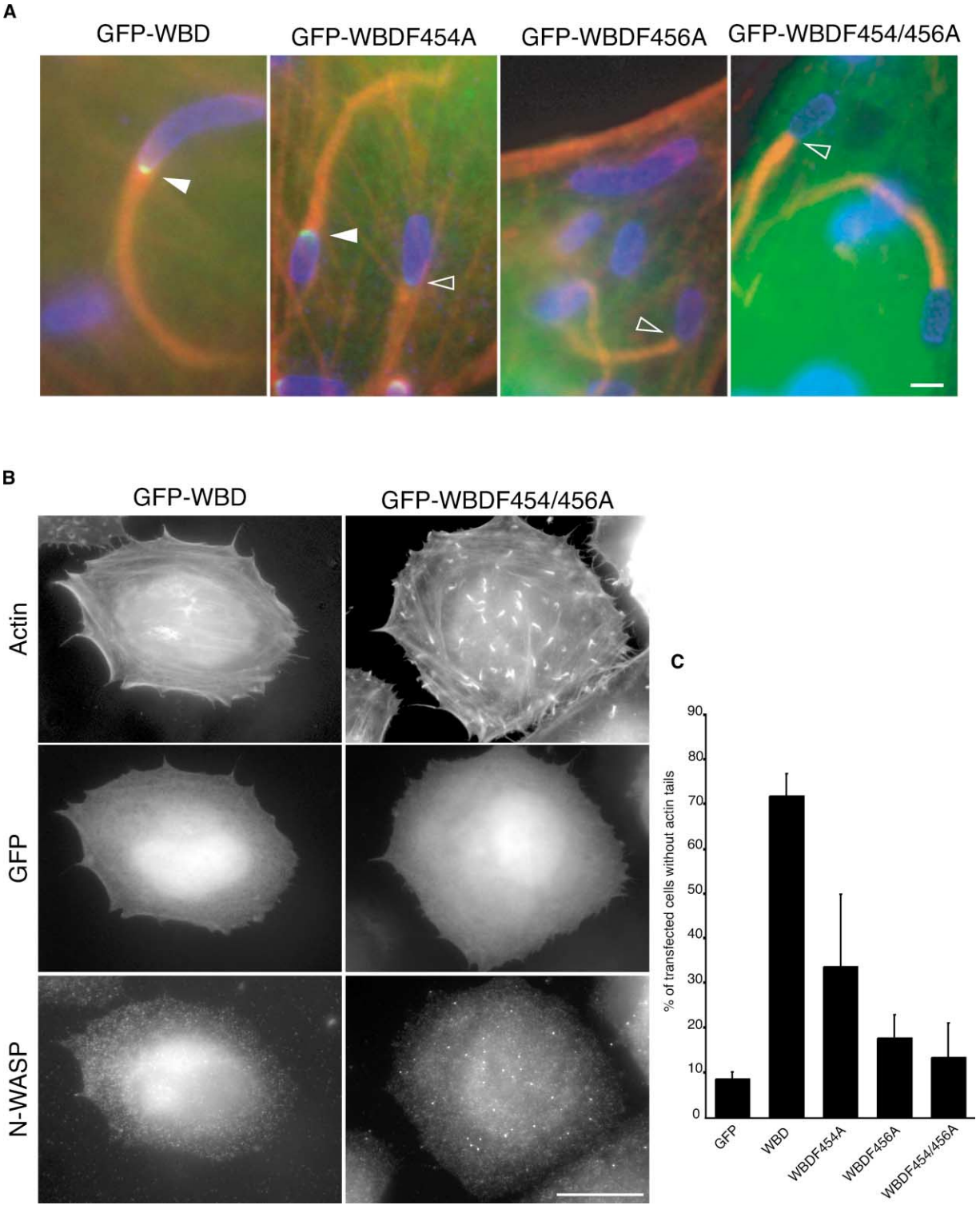

Figure 3. Analysis of the Effect of Mutation of Phenylalanine 454 and 456 in the WASP Binding Domain of WIP In Vivo

(A) Immunofluorescence images showing the localization of GFP-tagged mutant constructs of WIP-WBD (green), as indicated above the panels, to *Shigella* (blue) nucleating actin tails (red) in infected cells. GFP-WBDF454A, in contrast to GFP-WBD, is largely not recruited to *Shigella* (open arrowhead). In rare cases, however, as shown here GFP-WBDF454A is localized to the bacterium (filled arrowhead). In contrast, GFP-WBDF456A and GFP-WBDF454/456A are never recruited to the bacterium (open arrowheads). The scale bar represents 5 μm.

(B) Immunofluorescence images showing that, in contrast to GFP-WBD, expression of GFP-WBDF454/456A does not block the recruitment of endogenous N-WASP to vaccinia and actin tail formation. The scale bar represents 20 μm.

(C) Quantification of vaccinia-induced actin tail efficiency in cells overexpressing the indicated GFP construct. The data represent the average from four independent experiments, and error bars represent standard deviations.

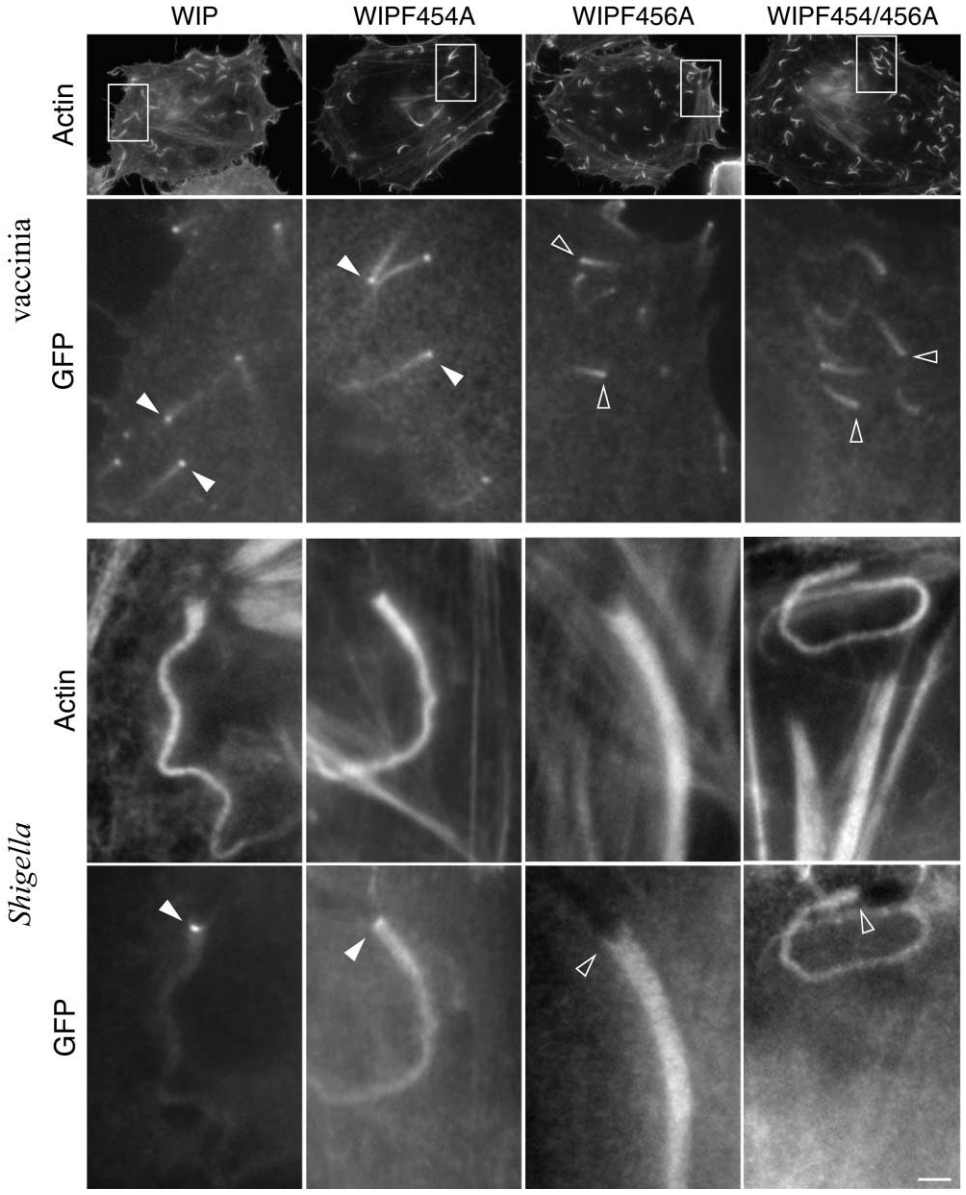

Figure 4. Interaction with N-WASP Is Required for Recruitment of WIP to Vaccinia and *Shigella*

Immunofluorescence images of cells infected with vaccinia or *Shigella* expressing the GFP-WIP mutants F454A, F456A, and F454/456A. GFP-WIP, and to a lesser degree GFP-WIP-F454A, are recruited to vaccinia or *Shigella* nucleating actin tails (filled arrowheads). In contrast, GFP-WIP-F456A and GFP-WIP-F454/456A are not recruited to the pathogen surface (open arrowheads) but are observed down the actin tail. The scale bar represents 2 μm.

to 13.5% ± 7.6% when both mutations were combined (Figure 3C). Consistent with its lack of inhibition of actin tails, GFP-WIP-WBDF454/456A did not block recruitment of N-WASP to vaccinia (Figure 3B). Taken together, our in vivo data using the *Shigella* and vaccinia pathogen systems confirm that we have identified the N-WASP WH1 binding motif in WIP.

Observations by Snapper et al. have demonstrated that, in the absence of N-WASP, vaccinia is unable to recruit WIP [18], which is consistent with the suggestion that both proteins are recruited as a complex [1]. While this study formally shows that N-WASP is essential for actin-based motility of vaccinia, it does not rule out the possibility that additional WH1-independent interactions might also contribute to WIP recruitment and stabilization of the complex on the virus. To explore this possibility, we examined the localization of full-length WIP containing the phenylalanine 454 and 456 alanine substitutions in *Shigella* and vaccinia-infected cells. We found that GFP-WIP-F454A was still recruited to *Shigella* and vaccinia nucleating actin tails, albeit at reduced levels when compared to GFP-WIP, and this finding is consistent with weaker binding to the N-WASP WH1 domain in vitro (Figure 4). GFP-WIP-F456A or double phenylalanine mutants were never recruited to either *Shigella* or vaccinia (Figure 4). It was noticeable that all

GFP-WIP mutants had a more pronounced association along the length of the actin tail than wild-type WIP. This was especially apparent in the case of *Shigella*-induced actin tails (Figure 4). This localization is consistent with the observation that the protein is associated with actin stress fibers in vivo and can bind actin filaments in vitro [2, 19]. Taken together, our observations using WIP mutants indicate that, while they still bind Nck (data not shown), their ability to interact with the WH1 domain of N-WASP is essential for recruitment to both pathogens. In addition, the association of GFP-WIP mutants deficient in WH1 binding with the actin cytoskeleton in vivo suggests that additional N-WASP-independent roles must also exist for WIP, as previously suggested by Vetterkind et al. [19]. Future studies must address what these roles are and whether WIP as well as the related CR16 and WICH act as adapters or play a more active regulatory function when they are complexed to N-WASP or WASP.

Supplementary Material

Supplementary Material including detailed Experimental Procedures is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

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